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Evaluation of potential genetic and chemical markers for Scots pine tolerance against *Heterobasidion annosum* infection

Mukrimin Mukrimin^{1,2} · Andriy Kovalchuk¹ · Rajendra P. Ghimire³ · Minna Kivimäenpää³ · Hui Sun^{1,4} · Jarmo K. Holopainen³ · Fred O. Asiegbu¹

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Abstract

Main conclusion Two terpene compounds and four genes were identified as potential biomarkers for further evaluation for Scots pine susceptibility or tolerance against *Heterobasidion annosum*.

Abstract Scots pine (*Pinus sylvestris*) is one of the main sources of timber in the boreal zone of Eurasia. Commercial pine plantations are vulnerable to root and butt rot disease caused by the fungus *Heterobasidion annosum*. The pathogen affects host growth rate, causes higher mortality and decreases in timber quality, resulting in considerable economic losses to forest owners. Genetic and biochemical factors contributing to Scots pine tolerance against *H. annosum* infection are not well understood. We assessed the predictive values of a set of potential genetic and chemical markers in a field experiment. We determined the expression levels of 25 genes and the concentrations of 36 terpenoid compounds in needles of 16 Scots pine trees randomly selected from a natural population prior to artificial infection. Stems of the same trees were artificially inoculated with *H. annosum*, and the length of necrotic lesions was documented 5 months post inoculation. Higher expression level of four genes included in our analysis and encoding predicted α -pinene synthase (two genes), geranyl diphosphate synthase (GPPS), and metacaspase 5 (MC5), could be associated with trees exhibiting increased levels of necrotic lesion formation in response to fungal inoculation. In contrast, concentrations of two terpenoid compounds, β -caryophyllene and α -humulene, showed significant negative correlations with the lesion size. Further studies with larger sample size will help to elucidate new biomarkers or clarify the potential of the evaluated markers for use in Scots pine disease resistance breeding programs.

Keywords Chemical defences · Gene expression · *Heterobasidion* · Root rot disease · Scots pine · Terpenoid

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00425-019-03270-8>) contains supplementary material, which is available to authorized users.

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Abbreviations

GPPS	Geranyl diphosphate synthase
MC5	Metacaspase 5
PCD	Programmed cell death
PCoA	Principal coordinates analysis
PERMANOVA	Permutational multivariate analysis of variance

Introduction

Scots pine (*Pinus sylvestris* L.) is a dominant tree species, occupying 64% of Finnish land area, and the largest growing stock, reaching 1157 million m³. It is a species of major economic importance, widely used in timber, pulp, and paper production (Metla 2013). A health status of Scots pine trees is an essential prerequisite for the sustainable timber production. In fact, potential threats to Scots pine stands include

both biotic (e.g., pathogenic fungi, bacteria and nematodes, herbivorous insects and mammals, natural competition, and human interference), and abiotic factors (e.g., drought, wind-fall, etc.) (Metla 2013). The dynamics of forest ecosystems, including their productivity and economic value, is significantly affected by fungal pathogens. In Northern Hemisphere forests, fungi comprising *Heterobasidion annosum* s.l. species complex cause root-rot disease of conifer trees (Asiegbu et al. 2005). The trees infection by *H. annosum*, which attacks the sapwood and kills the vascular cambium, causes decreased growth and timber quality, and eventually leads to mortality of trees (Swedjemarm and Stenlid 1995; Oliva et al. 2011; Gori et al. 2013).

The species *H. annosum*, *H. parviporum*, and *H. abietinum* are European representatives of the species complex *H. annosum* s.l., attacking particular conifer hosts comprising Scots pine, Norway spruce (*Picea abies*), and silver fir (*Abies alba*), respectively (Asiegbu et al. 2005; Garbelotto and Gonthier 2013).

Scots pine, like other plants, possesses defence mechanisms that provide protection against damage caused by biotic factors and environmental stresses. Constitutive tree defences deter pests and pathogen entry and invasion due to the presence of the physical barriers, e.g., lignified cell walls, waxy epidermal cuticle, and suberized bark as well as defensive chemicals (Franceschi et al. 2005). In contrast, inducible tree defences are activated in response to pathogen invasion, and include, among other factors, cell wall reinforcement, oxidative burst, and induction of certain biochemical pathways involved in the production of defensive chemicals, such as phenolics, terpenoids, and alkaloids (Kovalchuk et al. 2013). Certain chemical compounds may impart resistance to herbivorous insects and their associated pathogens (Keeling and Bohlmann 2006).

One of the common responses of conifer trees to fungal and insect attacks is an increased production of oleoresin, which is a complex mixture of volatile mono- and sesquiterpenes and diterpenoid resin acids (Napierala-Filipiak et al. 2002). Components of oleoresin display antifungal, antibacterial, or anti-insect properties. The composition of terpenoid compounds produced by individual trees show considerable variability within populations, allowing the recognition of so-called chemotypes. Chemotypes of Scots pine show remarkable differences in the proportions of produced monoterpenes, particularly α -pinene and δ -3-carene (Thoss et al. 2007; Bäck et al. 2012). Importance of terpenoid compounds in defense against pathogens and pests was previously demonstrated in numerous studies (Köpke et al. 2010; Ott et al. 2011; Roach et al. 2014). However, the contribution of individual terpenoid components may vary in different pathosystems. In Rocky Mountain lodgepole pine (*Pinus contorta* var. *latifolia*), terpene δ -3-carene was found to be induced to higher level than β -pinene, which provided a

greater level of defence against mountain pine beetles (*Dendroctonus ponderosae*) (Ott et al. 2011). In another study, (+)- α -pinene together with pinosylvin monomethyl ether were detected in the stems of Scots pine and were induced by the presence of the fungi *Ophiostoma brunneo-ciliatum* and *Hyalorhinocladia macrospora* (Villari et al. 2012). In addition, an earlier finding revealed that the terpene concentration in Scots pine trees highly susceptible to *H. annosum* was significantly higher compared to less susceptible trees, while δ -3-carene concentration was negatively correlated with lesion length in trees (Keriö 2015). Stilbene synthase also plays a role in the induced responses of Scots pine to fungal infection of *H. annosum* (Kovalchuk et al. 2017).

Plants including trees have immune defense mechanisms through sacrificing their infected cells for the benefit of the remaining cells, primarily aiming against infections caused by biotrophic pathogens (Jaber et al. 2014). Tree defense comprises two classes, namely resistance and tolerance. Resistance is the ability to reduce growth and infection of the pathogen, whereas tolerance is the ability to reduce or offset its negative performing such that trees can still grow and produce biomass and volume normally (Katjiua and Ward 2006; Sniezko and Koch 2017). In contrast to pathogenesis caused by necrotrophic pathogens, the mechanisms, usually known as hypersensitive response and programmed cell death (PCD), allow plants to prevent spread of biotrophic pathogens during an early stage of infection; however, hypersensitive response and PCD are not known to occur in necrotrophic root/butt diseases of forest trees (Han 2019). In contrast to previous studies, our study objective was to explore the possibility to identify tolerant trees based on their inherent genetic and chemical properties that are not dependent on chemicals or genes induced due to artificial inoculation.

We tested the hypothesis that the composition of terpenoid compounds in the needles of Scots pine trees can be used as a biochemical marker for predicting the level of tolerance to *H. annosum* infection. It was demonstrated previously that, despite pronounced differences in terpene content between needles and wood of Scots pine, the concentrations of many terpenoids in these tissues show a significant correlation (Manninen et al. 2002). This observation justifies our approach of testing concentrations of needle terpenoids as potential biochemical markers for tolerance against stem pathogen.

Furthermore, we assessed the expression level of selected genes involved in terpene, stilbene, and flavonoid biosynthesis and PCD in Scots pine trees with varying levels of tolerance to the pathogen. A primary motivation for the study was a need for the identification of reliable predictive markers that would allow estimation of tree tolerance to *H. annosum* infection in a non-invasive way, without performing artificial inoculations. Availability of such markers will greatly

facilitate screening for trees with high tolerance to root disease, while decreasing the required cost and labor. Since we collected tree samples before inoculation for terpene compounds and gene expression analysis, we emphasize that this work is distinct from other studies (Zamponi et al. 2007; Madmony et al. 2018) where samples were collected post-inoculation. When trees are wounded, the production of secondary metabolic compounds is induced, which interferes with the identification of trees that are naturally genetically resistant. The primary objective is to explore the potential to identify markers for those tolerant trees that had inherent genetic make-up with constitutive gene expression that does not depend on pathogen induction.

Materials and methods

Study site and sample collection

The 10- to 15-year-old Scots pine (*P. sylvestris* L.) trees at a Research Forest site in Lapinjärvi (Uusimaa region, Southern Finland) were used in this study. The geographic position of the study site is 60°39' 6"N, 26°8'17"E and its elevation is ca. 57 m above sea level. Collection of needle samples (second year) and artificial tree inoculation were performed on June 30, 2016. Before the inoculation of selected trees, needle samples were collected for RNA extraction and terpenoid analysis. These samples were kept on dry ice during sample harvesting and transportation, followed by storage at −80 °C until used for analyses.

Preparation of fungal inoculum

The Scots pine wood dowels (7 mm height, 10 mm diameter) colonized by *H. annosum* were used as the inoculum. The dowels were moistened with water (20 ml/200 dowels) and autoclaved in glass jars for 20 min. The sterile dowels were placed on 2% malt extract agar (MEA) plates to be pre-colonized by a heterokaryotic isolate of *H. annosum* (isolate 06068), followed by incubation for 3–4 weeks. Autoclaved dowels placed on sterile MEA plates were used as a control for mock inoculations.

Inoculations, sample harvesting, and measurement

The inoculation method used (Fig. 1) was similar as previously described (Keriö et al. 2014; Mukrimin et al. 2018). The stem surface was sterilized with 70% ethanol followed by making a hole with a 70% ethanol-sterilized puncher (10-mm diameter) through the tree bark to remove the rhytidome, phellem, and cambium. Inoculation spots were made at two spots on the stems of selected trees at 50 cm and 100 cm above ground level. The *H. annosum*-colonized dowels of the same diameter as the holes were placed in the holes and then covered with Parafilm M® (Sigma-Aldrich). For the control trees (mock inoculation), holes were filled with autoclaved wood dowels. The trees were harvested for phenotyping measurement 5 months post inoculation and stored at −20 °C until used. There were 16 trees used for inoculation treatment and six trees for wounding or mock treatment.

Fig. 1 Illustration of inoculation and measurement method used in Scots Pine trees experiment in the field

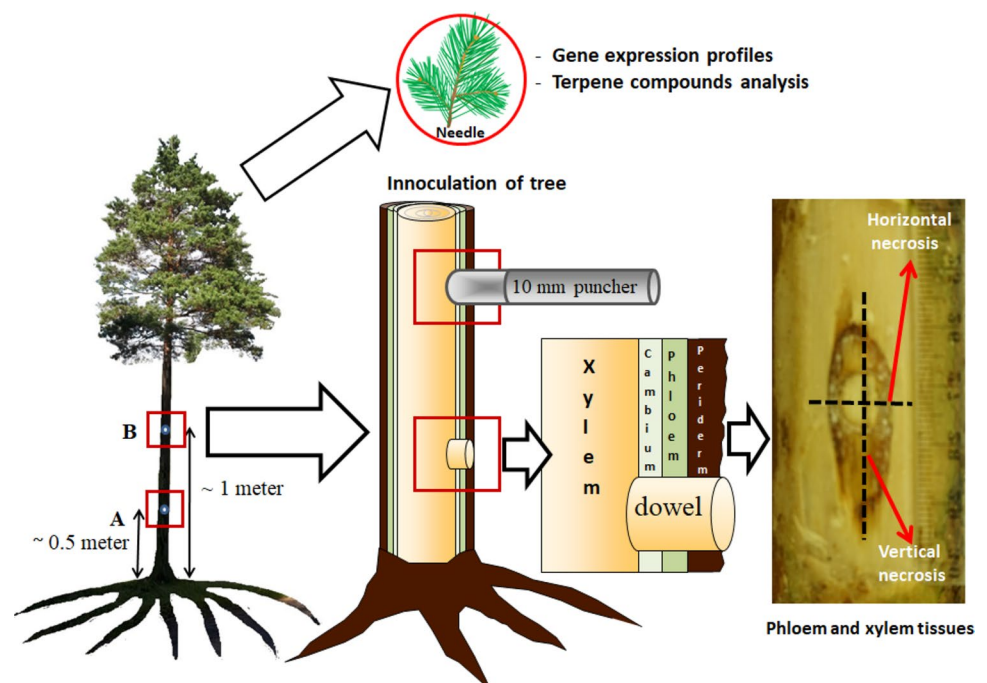


Table 1 List of primer sequences used for RT-PCR analysis in this study

Primer	Primer sequence	GenBank, ConGenIE and PtNewbler1 accession numbers	Predicted gene function	Amplicon length, bp	References
PsTPS5_F	gcttctactgcctgcgtctt	GU248335.1	Farnesene synthase	64	Köpke et al. (2010)
PsTPS5_R	cgctgttcagggaattgaagg				
PcTPS_3Car1_F	tgtctcttatttccgctgtgc	JQ240307.1	3-carene synthase 1	70	Hall et al. (2013)
PcTPS_3Car1_R	tcacgaacagaactgatcaaga	JQ240305.1			
PbTPS_3Car2_F	tgtctgtggagatgggtgaa	JQ240306.1	3-carene synthase 2	60	Hall et al. (2013)
PbTPS_3Car2_R	acgatgccgaatgaaggta				
PbTPS_(+)apin_F	gccgtctatgtaggccaaaac	JQ240298.1	(+)- α -pinene synthase	78	Hall et al. (2013)
PbTPS_(+)apin_R	aattcaacgggacagcaga				
PcTPS_(-)apin_F	ctgtgatctcgttgcttcc	JQ240303.1	(-)- α -pinene synthase	73	Hall et al. (2013)
PcTPS_(-)apin_R	gggttaagctcagcaccaga				
isotig12807_F	tcccagagaaaatcatgaatcc	isotig12807	GPPS (geranyl diphosphate synthase)	69	Keriö (2015)
isotig12807_R	agaacaggaggaaacccgtt				
isotig12811_F	tttgattactgacaaggccactt	isotig12811	GGPPS (geranylgeranyl diphosphate synthase)	78	Keriö (2015)
isotig12811_R	cccaacaactcgacagca				
isotig44391_F	ctcccaaacacctgtgat	isotig44391	GGPPS (geranylgeranyl diphosphate synthase)	64	Keriö (2015)
isotig44391_R	gcctgtatggacaatgacg				
isotig45191_F	gcatcatccctgaccattg	isotig45191	GPPS (geranyl diphosphate synthase)	68	Keriö (2015)
isotig45191_R	tcctcatccaagcctttacg				
17788-f	gctctcagaatgtcgaagca	isotig17788	Terpene synthase	60	Kovalchuk et al. (2015)
17788-r	gaaaccagacaccaatgttcc				
13137-f.3	gttacttagacccgacagc	isotig13137	α -pinene synthase	108	Keriö (2015)
13137-r.3	gcaacactgaagccatctc				
17929-f.2	ttctcgagcacgtcctc	isotig17929	α -pinene synthase	97	Keriö (2015)
17929-r.2	tggtagcagcgcgtatc				
PsTPS6_F	ttctgcagcaaatattctcc	EF679330.1	caryophyllene/humulene synthase	69	Köpke et al. (2008)
PsTPS6_R	cgtcgatgagctgattgtca				
PsTPS7_F	tgggaatttctaaagcatgaca	EF679331.1	1(10),5-germacradien-4-ol synthase	70	Köpke et al. (2008)
PsTPS7_R	tgccctgtgcaagattga				
STS_F	tccgactggaacaagtgttc	X60753.1	pinosylvin synthase	73	Kovalchuk et al. (2017)
STS_R	gcttgccctcccccgaatcaag				
CHS_F	atggctgcaggaatgatgaagg	X60754.1	chalcone synthase	87	Kovalchuk et al. (2017)
CHS_R	agtccaatagcgaggatg				
PaRNSI1-f	ggacgcttctactgttcaaatg	MA_95383g0020	RNSI1 (ribonuclease RNS1)	88	This study
PaRNSI1-r	atcgttgtcccagaaaggagg				
PaRNSI2-f	cagagcaacagttgtagattgcag	MA_34759g0010	RNSI2 (ribonuclease RNS1)	70	This study
PaRNSI2-r	aaggcggtgtccataagccctc				
PaCEP-f	agaactcatgggtaccgactg	MA_22695g0010	CEP (cysteine endopeptidase CEP)	90	This study
PaCEP-r	cgtttatagcgcacacaccataagg				
PaXCP-f	atcgttagcttgcggcagcag	MA_10433186g0010	XCP (xylem cysteine peptidase XCP1)	68	This study
PaXCP-r	aagtgcctgtcttcccactc				
PaXBCP-f	gcagaggagaagcaacagag	MA_10429635g0010	XBCP (xylem bark cysteine peptidase XBCP3)	92	This study
PaXBCP-r	ccaagccagtagctcagggtc				
PaBFN-f	ctgagtatgcagagggtgatcttg	MA_75204g0010	BFN (endonuclease BFN1)	93	This study
PaBFN-r	caatgaagtgtaatggacttgaccag				
PaMC5-f	ggaagcccatcagcaacagg	MA_10430487g0010	MC5 (metacaspase 5)	112	This study
PaMC5-r	gcagcatcggcgttattgtcac				

Table 1 (continued)

Primer	Primer sequence	GenBank, ConGenIE and PtNewbler1 accession numbers	Predicted gene function	Amplicon length, bp	References
37123-f	agttgactttccatccaacc	isotig37123	α -/ β -pinene synthase	84	Keriö (2015)
37123-r	ctgtcctcctgataacatc				
PsTPS3_F	gctgggaacgatacattgaag	EF679332.1	longifolene/longipinene synthase	72	Köpke et al. (2008)
PsTPS3_R	caaacgtaggacatgctcgag				
EF1-a_F	caccttgggagtgaagcaaatg	<i>EF1-a</i>	elongation factor 1 α	58	Vestman et al. (2011)
EF1-a_R	gggagtagtgccatccatcttg				
isotig44517_F	gggagttggcatcatgagtt	isotig44517	Histone H2A	66	Keriö (2015)
isotig44517_R	gactttgaagacttcgctttcc				

Phenotyping measurements and statistical analysis

The necrotic lesions in phloem and xylem tissues were measured for both horizontal and vertical dimensions after removing the periderm tissues using a sterilized knife. The stem diameter was also documented. Based on phenotypic lesion-size data, trees were separated into two stringency levels (high and low) and further grouped as susceptible, moderate, or tolerant (Suppl. Table S1). Applying the low stringency level, tree samples having lesion size of < 120 mm were categorized as tolerant trees, lesion size of 120–180 mm—as the intermediate trees, and the lesion size of > 180 mm—as the susceptible. Using the high stringency level, tree samples having lesion size of < 106 mm were categorized as tolerant trees, lesion size of 106–240 mm—as the intermediate trees, and the lesion size of > 240 mm—as the susceptible. A one-way analysis of variance (ANOVA) was performed to detect differences among the necrotic lesion sizes between the inoculation and wounding treatments and between groups for gene expression level and terpene compounds. The growth rates of *H. annosum* were assessed by *t* tests for two-sample assuming unequal variances. Pearson's correlation analysis was used to determine correlations between the stem diameter and the sizes of necrotic lesions (sum of all necrosis, horizontal and vertical lesions for both phloem and xylem). Differences were considered as statistically significant if *P* value was below the threshold of 0.05. Phenotyping data were analyzed using the SPSS version 25.0 (IBM Corporation).

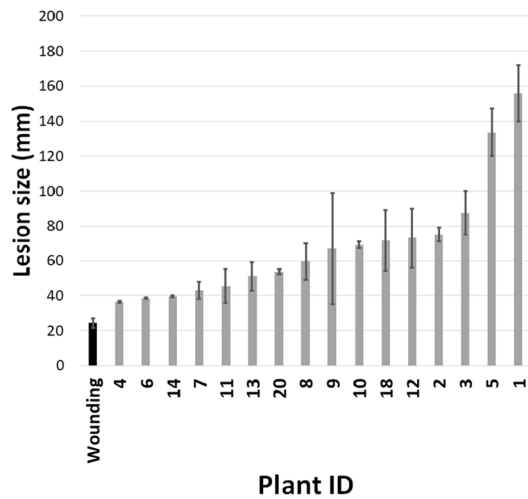
RNA extraction, cDNA preparation, and RT-PCR analysis

RNA was extracted from the Scots pine needles as described previously (Chang et al. 1993) with some modification. In brief, Scots pine's needles were grounded using an IKA® A11 basic mill (IKA-Werke, Staufen, Germany). The sample (2 g) was transferred to a 50 ml sterile tube and resuspended in 10 ml extraction buffer (2% CTAB, 2% PVP,

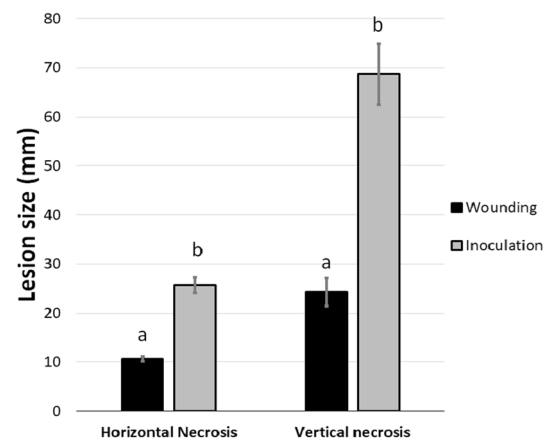
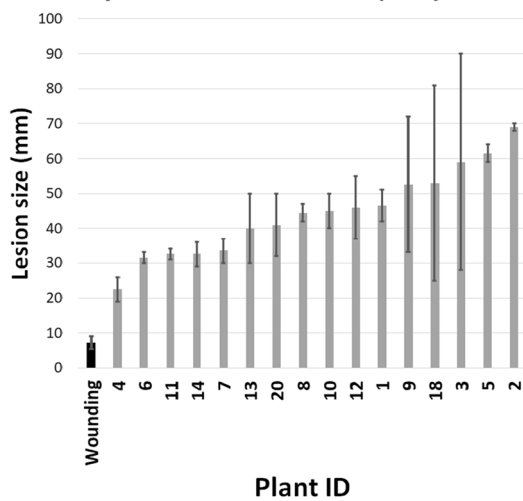
100 mM Tris–HCl, 25 mM EDTA, 2 M NaCl, and nuclease-free water) and 200 μ l of mercaptoethanol (pre-warmed at 65 °C). After vortexing, the mixture was incubated for 6 min at 65 °C. 10 ml of chloroform-isoamyl alcohol (24:1, v/v) was added and mixed and centrifuged at 10,000g at 24 °C. 9 ml of supernatant and an equal volume of chloroform-isoamyl alcohol (24:1, v/v) were transferred to a new 50 ml sterile tube. After centrifugation at 10,000g for 10 min at 24 °C, 8 ml of supernatant was transferred to a new 50 ml sterile tube and added with 2 ml of 10 M LiCl, followed by precipitation at 4 °C overnight. The solution was centrifuged at 10,000g for 30 min at 4 °C and the supernatant was pipetted out carefully. After drying, 200 μ l of cold 70% ethanol was added to the pellet and then centrifuged at 10,000g for 5 min at 4 °C. The supernatant was then pipetted out and the pellet was dried out. The pellet was suspended in 100 μ l of nuclease-free water and RNA samples were stored at –80 °C.

Each RNA sample (1 μ g) was treated with DNase I (Thermo Scientific), and cDNA was synthesized by ReverTaid Reverse Transcriptase (RT) (Thermo Scientific) according to the manufacturer's instructions. In this study, 25 primer pairs were used (Lorenz et al. 2012; Nystedt et al. 2013; Keriö 2015; Sundell et al. 2015) (Table 1).

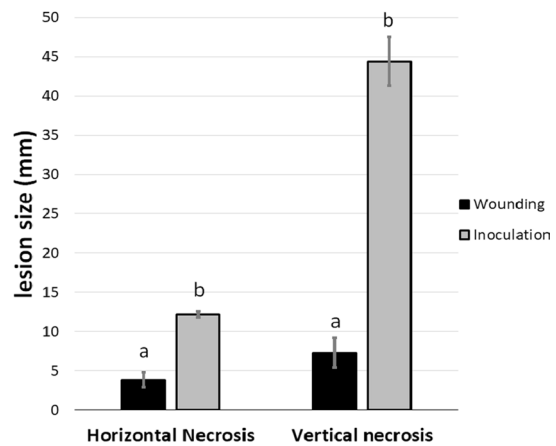
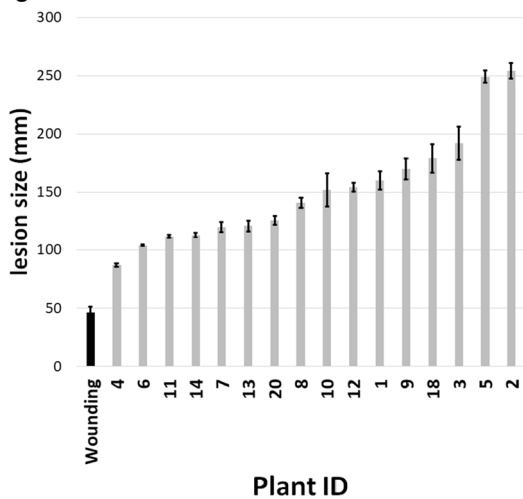
qPCR reactions were carried out using the LightCycler 480 II instrument (Roche). Two technical replicates were carried out and each reaction was performed as follows: 5.5 μ l (11 ng) of cDNA, 1 μ l forward primer (final concentration 0.67 μ M), 1 μ l reverse primer (final concentration 0.67 μ M) and 7.5 μ l Master-Mix (Roche). The following cycling parameters were applied: initial pre-incubation at 95 °C for 5 min followed by 45 amplification cycles (10 s at 95 °C, 10 s at 55 °C and 10 s at 72 °C). A final melting curve analysis was also included to assess the primer specificity. The crossing points (Cp) values were calculated with the second derivative method using the Roche LightCycler 480 software (version 1.5.1.62). Primer efficiencies were applied in the calculation of the relative expression level of each gene with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001)

a Phloem Vertical necrosis (mm)

Necrosis of Scots Pine's phloem

**b** Xylem Vertical necrosis (mm)

Necrosis of Scots Pine's Xylem

**c** sum of all lesions (mm)

sum of all lesions

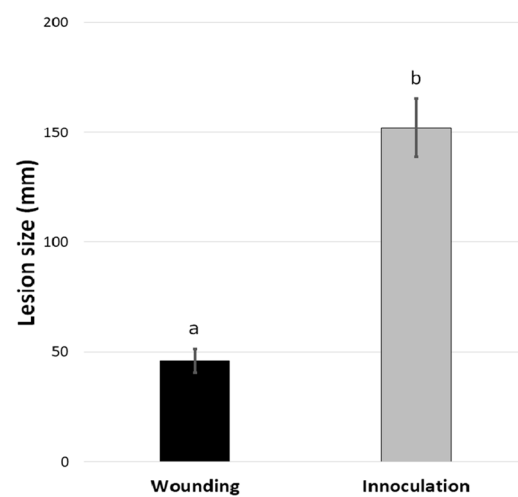


Fig. 2 Mean of measurement result of **a** phloem vertical necrosis, **b** xylem vertical necrosis, **c** sum of all lesions for wounded and inoculated trees showing significant difference at $P < 0.005$. The error bar is reflected as SE

to estimate the relative expression values of the genes in the sampled trees. The genes encoding elongation factor 1 alpha (EF-1 α) (Vestman et al. 2011) and histone H2A (Keriö 2015) were used as reference genes, but histone H2A had low expression level and was excluded from the analysis. The crossing points (Cp) values from the RT-PCR were imported and analyzed in SPSS Statistics 25 software (IBM).

Terpenoids analyses

Terpenoids were analyzed from needle samples of Scots pine using previously published methods (Kainulainen et al. 1992). Needle samples were cut into small pieces under N₂ and 200 mg of samples were extracted in 2 ml of *n*-hexane at room temperature for 2 h and washed twice with 2 ml *n*-hexane. 1-chloro-octane was used as an internal standard. The extracts were analyzed using an Agilent 7890B gas chromatograph equipped with a mass-selective detector (type 5977A). Separations were carried out on a 30-m HP-5 ms Ultra Inert (i.d. 0.25 mm; film thickness 0.25 μ m, Agilent) column. Helium was used as carrier gas, and linear velocity was about 40 cm/s. The splitless (purge time off 1 min) sampling technique was used, and 1 μ l was injected. The column temperature was programmed from 50 to 115 °C at 5 °C/min, then to 280 °C at 15 °C/min and hold for 10 min. Mass numbers from *m/z* 33 to 350 were recorded. Compound identification and quantification were based on their mass spectra, retention time, and authentic standard compounds as described by Kainulainen et al. (1992). Limonene and β -phellandrene were coeluted.

Principal coordinates analysis (PCoA) was used to visualize the structure of the tolerant, intermediate, and susceptible trees based on the xylem vertical necrosis. Distance-based linear model (DistLM) analyses were performed to assess the correlation of the susceptible, intermediate, and tolerant trees with gene expression profiles and with monoterpene and sesquiterpene concentrations as variables in PRIMER v.6 (Clarke and Gorley 2006) with the add-on package of Permutational Multivariate Analysis of Variance (PERMANOVA)+ (Anderson et al. 2008).

Results

Variation in lesion length in response to *H. annosum* inoculation

At 5 months post infection, the vertical length of necrotic lesions in the inoculated Scots pine stems varied from

36.5 to 156 mm (68.7 ± 9.4 mm) and from 22.5 to 69 mm (44.4 ± 8.5 mm) in phloem and xylem, respectively. The total necrotic lesion size, based on sum of all lesions (phloem and xylem), varied from 87 to 254 mm (152.13 ± 12.07 mm). The lesion length was significantly larger in inoculated than in wounded trees ($P < 0.001$) for phloem vertical necrosis, xylem vertical necrosis, and sum of all lesions (Fig. 2).

The lesion length was normally distributed among the analyzed trees; hence, the inoculation treatment was classified into three groups according to the sum of all necrosis, namely tolerant, intermediate, and susceptible trees (Suppl. Fig. S1). Lesion sizes were significantly different ($P < 0.001$) among the groups and between inoculation and wounding. Based on Pearson's analysis, the tree diameter (growth trait) was significantly positively correlated with length of necrotic lesions in phloem (Table 2 and Fig. 3). In contrast, tree diameter showed a significant negative correlation with wounding horizontal necrosis in phloem (Table 2). In addition, xylem vertical necrosis showed a significant positive correlation with phloem horizontal, phloem vertical, xylem horizontal necrosis, and sum of all necrosis (Fig. 3).

Gene expression analysis

The relative gene expression is presented in Fig. 4a. We found that significant differences were evident among groups (tolerant, intermediate, and susceptible) in genes encoding α -pinene synthase for the sum of all necrosis measurements ($P = 0.012$), and (–)- α pinene synthase for xylem vertical necrosis ($P = 0.027$) (Suppl. Table S2). While for genes encoding geranyl diphosphate synthase (GPPS) and metacaspase 5 (MC5), no significant differences were observed among the groups ($P > 0.05$).

The results of correlation analysis are presented in Table 3 and Fig. 5. The expression level of a gene encoding predicted α -pinene synthase showed a positive correlation with the sum of all necrosis measurements (Fig. 5a). Correlation results for a putative (–)- α pinene synthase, a predicted GPPS, and MC5 are displayed in Fig. 5b–d and Suppl. Fig. S2. Trees with larger necrotic lesions were characterized by moderately increased transcript level of these three genes. Pearson's analysis showed that expression levels of a predicted α -pinene synthase gene had a positive correlation with the sum of all necrosis. Both a putative (–)- α pinene synthase gene and a predicted GPPS gene had a positive correlation with the size of xylem vertical necrosis, whereas the expression level of MC5 homologue had a positive correlation with the size of phloem horizontal necrosis (Table 3).

Terpene profiles of Scots pine needles

Results of analyses of terpenoid compounds in pine needles are presented in the Fig. 4b, c. The monoterpenes with

Table 2 Correlation coefficients between diameter and lesion size based on Pearson's analysis ($n=16$ for inoculated trees and $n=6$ for mock trees)

	Diameter
Inoculation	
Phloem horizontal necrosis	−0.101
Phloem vertical necrosis	0.530**
Xylem horizontal necrosis	−0.149
Xylem vertical necrosis	0.156
Mock inoculation	
Phloem horizontal necrosis	−0.542*
Phloem vertical necrosis	0.028
Xylem horizontal necrosis	−0.194
Xylem vertical necrosis	−0.242

Significant correlation at * $P < 0.05$ and ** $P < 0.01$

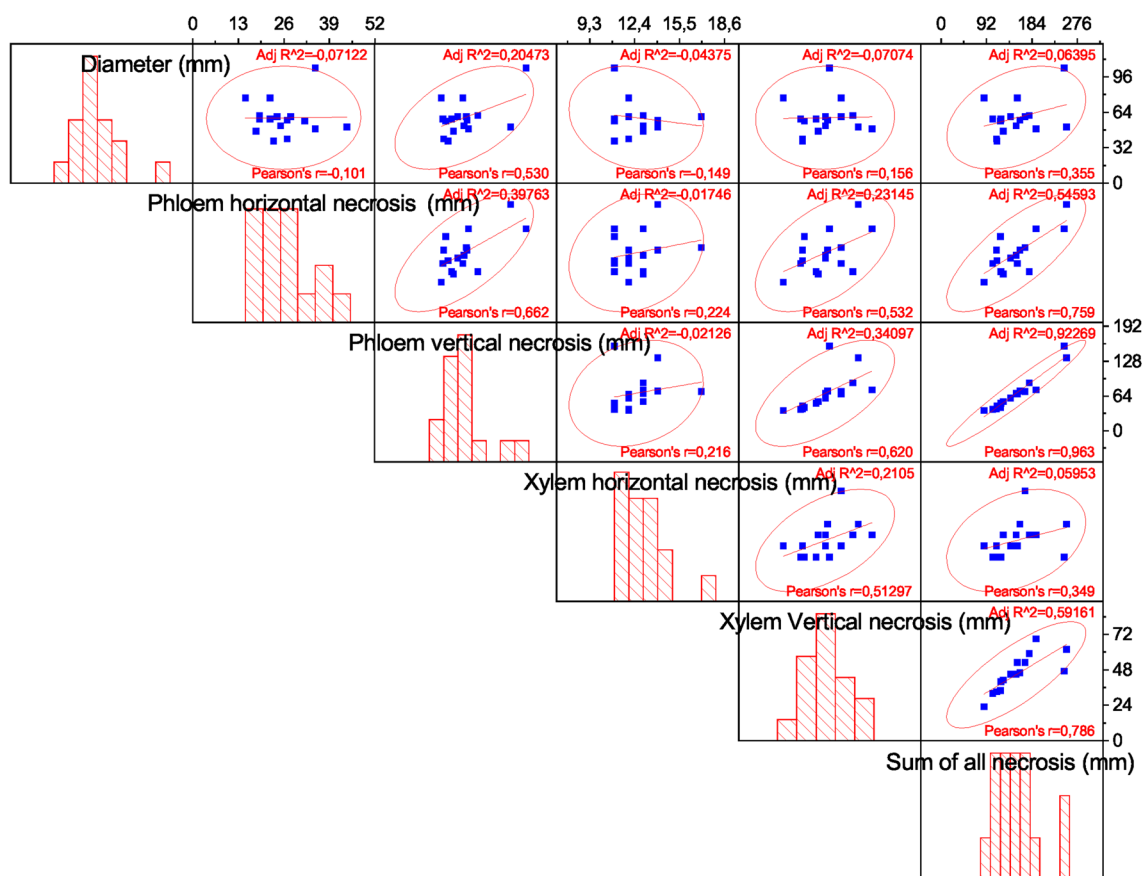
the highest concentrations were α -pinene and δ -3-carene (mean $> 1000 \mu\text{g/g}$ FW), the concentrations of remaining compounds were less than $250 \mu\text{g/g}$ FW. In the tolerant trees, α -pinene, limonene + β -phellandrene (co-eluted), trans- β -ocimene, and β -pinene had higher concentration compared

with other monoterpenes. Whereas, the highest concentration of monoterpenes in susceptible trees were δ -3-carene, bornyl acetate, tricyclene, sabinene, and α -thujene.

Among the analyzed sesquiterpenes, α -muurolene and β -elemene had the highest concentrations ($> 250 \mu\text{g/g}$ FW). In the tolerant trees, g-elemene had a higher concentration, compared with other sesquiterpenes. None of the analyzed mono- and sesquiterpenes compounds had significant difference among the groups ($P > 0.05$) (Suppl. Table S2).

Each lesion-length group varied in their total terpene concentration. We found that β -caryophyllene showed a negative correlation with sizes of phloem horizontal and vertical necrosis, and sum of all necrosis measurements; whereas α -humulene had negative correlation with the size of phloem vertical necrosis (Fig. 5c–h and Suppl. Fig. S3). Additionally, we found no significant differences among tolerant, intermediate, and susceptible trees in the total concentration of sesquiterpenes or monoterpenes ($P > 0.05$) (Suppl. Fig. S4).

PCoA and PERMANOVA analysis showed a clear separation between tolerant and susceptible trees (Fig. 6). We assessed a possible correlation of different factors (gene expression, mono- and sesquiterpenes) with the lesion size.

**Fig. 3** Scatterplot matrix of correlation between diameter and phloem horizontal, phloem vertical, xylem horizontal, and xylem vertical necrosis

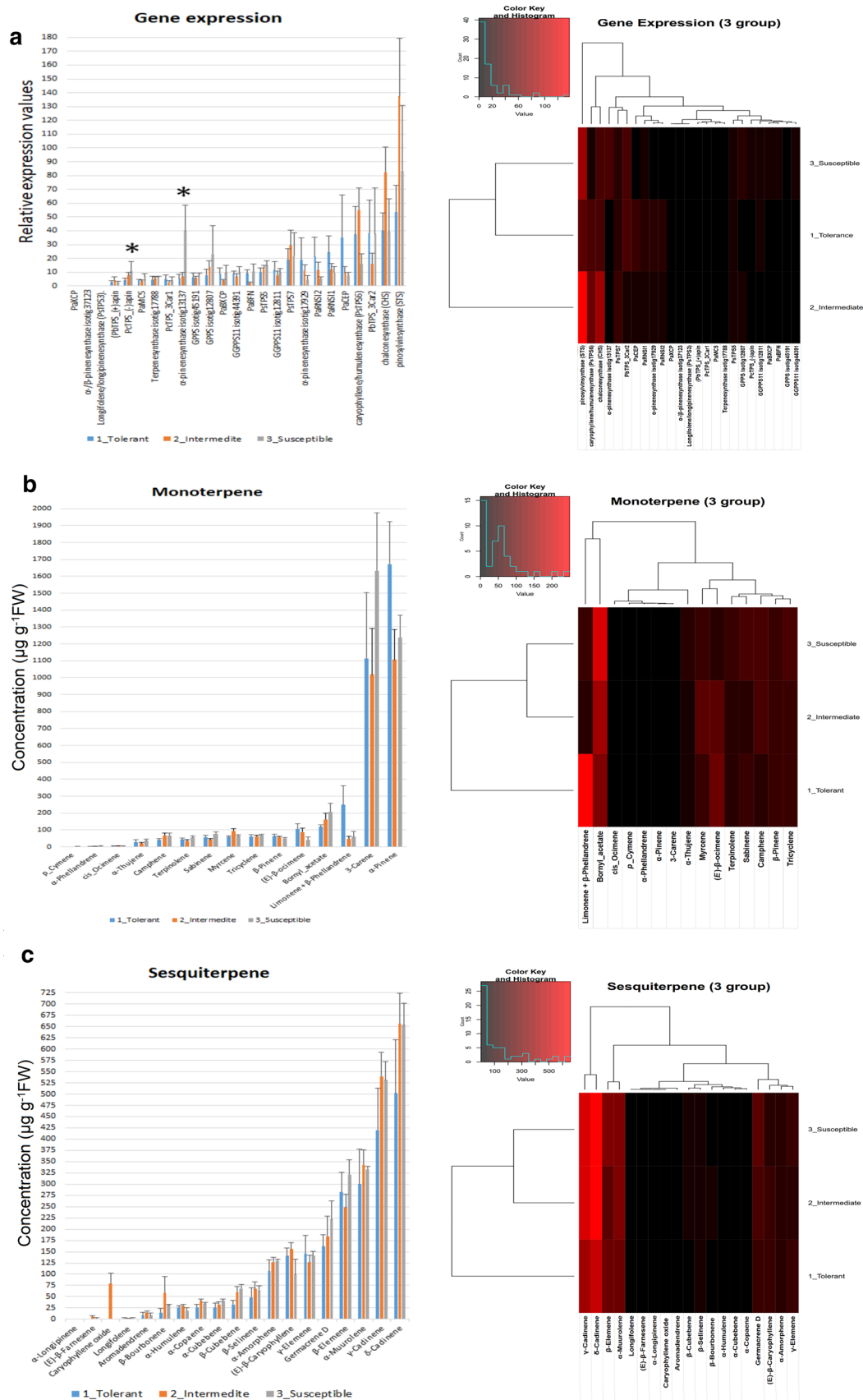


Fig. 4 Mean and heatmap for three group of trees based on sum of all necrosis. **a** Gene expression profiles. **b** Monoterpene compounds. **c** Sesquiterpene compounds

Table 3 Pearson correlation coefficients for gene expression and necrosis and for Sesquiterpenes and necrosis

		Sum of all necrosis (mm)
Based on the sum of all necrosis		
Gene expression		
α-pinenesynthase (isotig13137)		0.528*
Sesquiterpene (μg/gFW)		
β-Caryophyllene		−0.511*
	Phloem horizontal necrosis (mm)	Xylem vertical necrosis (mm)
Based on phloem and xylem necrosis		
Gene expression		
(−)-α pinene synthase (PcTPS_(-)apin)	0.088	0.529*
GPPS (isotig12807)	0.068	0.549*
MC5	0.586*	0.238
Sesquiterpenes (μg/gFW)		
β-Caryophyllene	−0.513*	−0.553*
α-Humulene	−0.480	−0.518*

Significant correlation at * $P < 0.05$ and ** $P < 0.01$

Among selected genes, only the gene encoding predicted α-pinene synthase (isotig13137) had a nearly significant expression level ($P = 0.056$). None of analyzed monoterpenes showed significant correlation with the lesion size. Among sesquiterpenes, β-caryophyllene was significantly correlated with lesion size ($P = 0.05$), whereas α-humulene was nearly significant ($P = 0.068$) (Suppl. Table S3).

Discussion

Despite the fact that field studies on root disease in natural forest ecosystem require more time than greenhouse studies (Swedjemarm and Stenlid 1996), artificial inoculations have been applied successfully in the field experiments to examine tree susceptibility to *Heterobasidion* infection (Swedjemarm and Karlsson 2004; Karlsson et al. 2008; Danielsson et al. 2011; Keriö et al. 2014). Although artificial inoculation followed by sampling of lesion areas is commonly done, relying solely on destructive inoculation to screen for resistant genotypes poses a technical and logistical challenge, which may be impractical for large-scale screening of novel biomarkers for durable resistance. One notable challenge is that chemicals induced due to destructive inoculation may be localized within the necrotic lesions. In this study, an alternative approach was explored. Samples for terpenoid and gene expression were collected prior to destructive inoculation. The results were later compared against necrotic lesions and used to identify genotypes that might be inherently tolerant or susceptible. Necrotic lesion size was used as a measure of host susceptibility or tolerance, while inoculation

and mock treatments were used for comparing differential responses of Scots pine trees to *H. annosum* infection.

The findings revealed that each individual Scots pine host genotype displayed a different degree of susceptibility to *H. annosum*. We found a significantly higher lesion size in inoculated treatments compared to mock inoculations, which is in accordance with a previously published work (Danielsson et al. 2011). Our study indicates that Scots pine trees with higher values of growth (diameter) showed considerably more sensitivity to *H. annosum* infection, particularly in phloem vertical necrosis. This result concurs with a previous result (Swedjemarm and Karlsson 2004), which clearly described that lesion length and fungal growth were positively correlated to diameter (growth trait) of *P. abies*. In contrast, (Karlsson et al. 2008) showed that stem diameter of *P. abies* had a strong negative correlation to both lesion size and fungal extension of *H. parviporum*. Another study revealed the diameter of Norway spruce saplings was significantly negatively correlated with lesion length particularly in xylem (Mukrimin et al. 2018).

Terpenoids are one of the largest group of defensive chemicals occurring in conifers (also found in broad-leaved trees), and this group is the most abundant compound class involved in chemical defense (Huber and Bohlmann 2006). Besides that, terpenoids are promising biochemical markers for characterizing of diverse tree species and for estimating geographic variation among different origins of *P. abies* (Persson et al. 1996). Composition of terpenoid compounds varies between different tissues (e.g., needles and wood) of the same tree. However, both proportional and absolute concentrations of individual compounds in needles and wood of Scots pine show significant correlations (Manninen

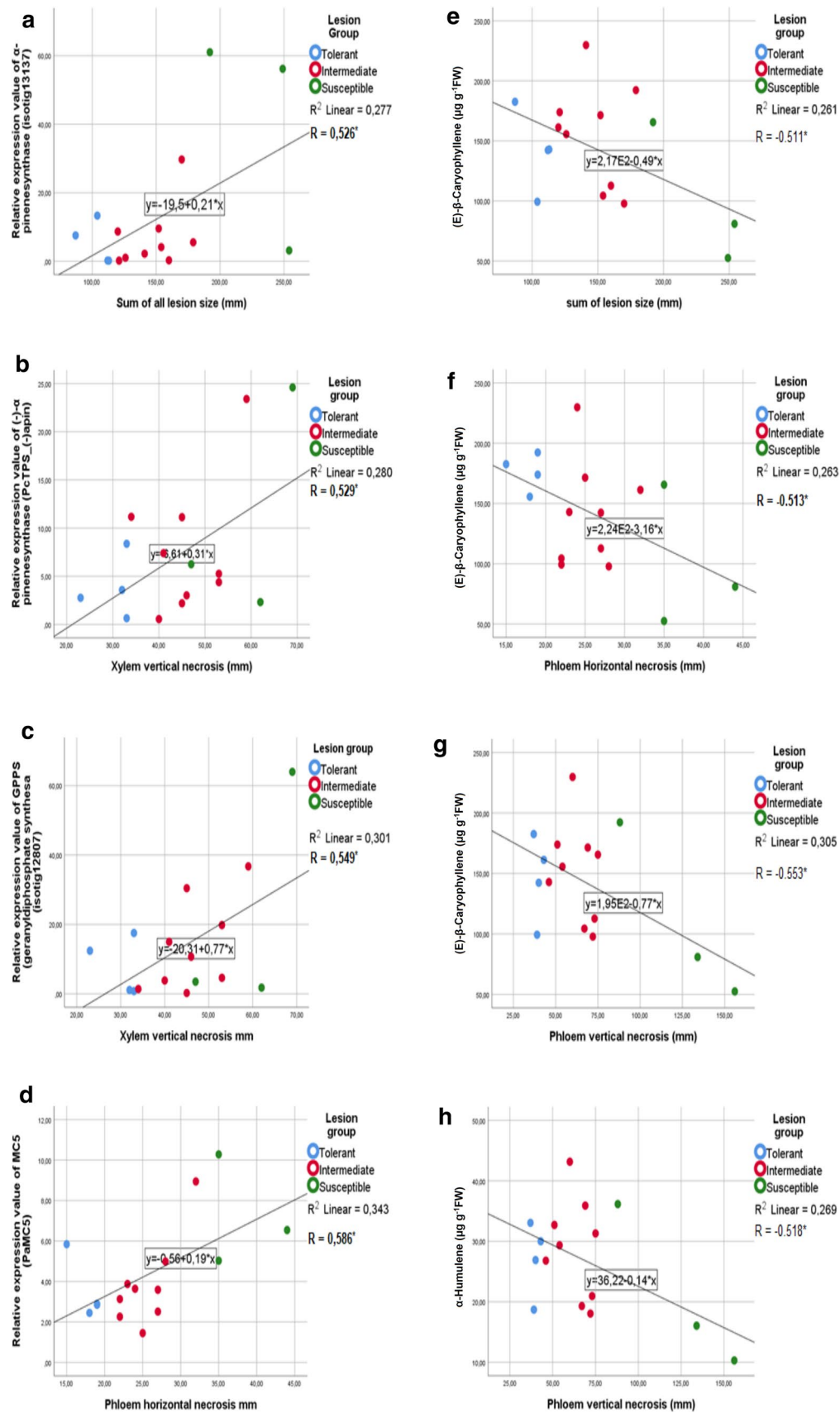


Fig. 5 Scatterplot of gene expression profiles and sesquiterpene compounds based on both sum of all necrosis and phloem and xylem necrosis

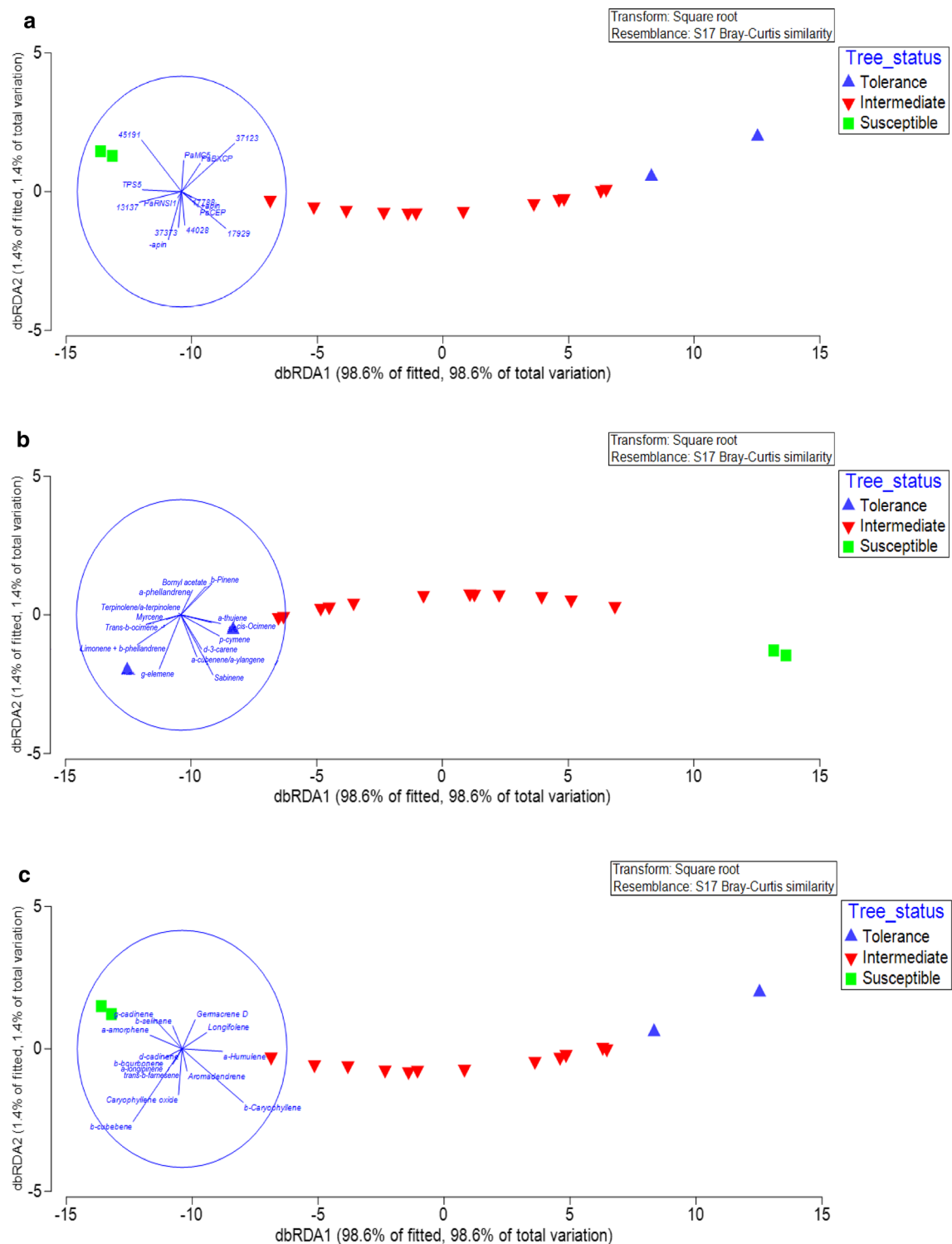


Fig. 6 Principal coordinates analysis (PCoA) based on the gene expression and terpene chemical compound relating to sum of all necrosis for samples from tolerance, intermediate, and susceptible

trees indicated with different symbols and colours. **a** Gene expression as factor. **b** Monoterpene as factor. **c** Sesquiterpene as factor

et al. 2002), supporting the idea of using needle terpene profiles as biochemical markers for resistance against stem pathogens. Our data demonstrated that the concentrations of

β -caryophyllene and α -humulene in Scots pine needles show significant negative correlation with the size of necrotic lesions developing in phloem in response to the inoculation

with *H. annosum*. Trees with lower concentrations of these two compounds were characterized by the largest necrotic lesions. At the same time, only two out of 36 assayed terpenoids showed correlation with the necrotic lesion size. This is in line with earlier reports showing lack of clear correlation between terpenoid content and resistance to wood-decay fungi (Nerg et al. 2004).

The high concentrations of terpene compounds recorded in the current study were similar to an earlier study. This study showed that δ -3-carene and α -pinene were the most abundant monoterpenes in Scots pine, which is supported by previous studies in which δ -3-carene and α -pinene were reported to be the most abundant monoterpenes in Scots pine (Vanhatalo et al. 2018) and white bark pine (Bullington et al. 2018). Other authors (Baradat and Yasdani 1988) revealed that Scots pine trees exhibited a strong broad-sense heritability of 3-carene, myrcene, limonene, and β -phellandrene under different environmental condition. Muona et al. (1986) found differences in 3-carene and other monoterpene compounds between natural stands and plus trees of Scots pine. Another previous study also found that the only detected sesquiterpene on the Scots pine seedling was β -caryophyllene (Tiiva et al. 2018). Whereas, the β -caryophyllene was the most abundant terpene of wood resin in maritime pine trees, either attacked or un-attacked by *Dioryctria sylvestrella* (Jactel et al. 1996). Another finding showed that Scots pine trees have increased release of terpenoids (β -caryophyllene and α -humulene) when pine common sawfly (*Diprion pini*) laid eggs on Scots pine needles (Köpke et al. 2010). These compounds (β -caryophyllene and α -humulene) have been shown to attract a biocontrol agent (*Closterocerus ruforum*) that parasitizes the sawfly eggs and plays a role in direct defense against sawfly larvae (Köpke et al. 2010).

The groups of tolerant and highly susceptible trees could not be reliably separated based on the expression pattern of the tested genes, despite some observed correlations of gene expression levels with the size of necrotic lesions. Within the sampled population, we observed considerable variation in the expression of many of the selected genes, which could be partly due to the genotypic differences of individual trees. Larger sample sizes may help identify statistically significant associations of expression level of certain genes with the degree of tolerance of Scots pine to *H. annosum* infection.

Despite studies of conifer defenses that showed δ -3-carene synthase expression has significant correlation to resistant trees (e.g., Fäldt et al. 2003; Roach et al. 2014), we found no support for this relationship in our study. Thus, the specific role of δ -3-carene synthase in the tree defense is still not well understood. It is also remains possible that increased levels of δ -3-carene or δ -3-carene synthase expression documented in lesion areas do not correlate to systemic or inherent abundance, or that chemotypic variation (e.g.,

α -pinene and δ -3-carene types) in Scots pine may mask other changes.

Our study identified four genes (α -pinene synthase (two genes), GPPS, and MC5) and two sesquiterpene compounds (β -caryophyllene, and α -humulene) suitable for further exploration as potential markers of tolerance/susceptibility of Scots pine trees to *H. annosum* infection. These data can provide additional support for future research on tree resistance to root and butt rot diseases. The role of the identified genes and compounds in the defense of pine trees deserves further investigation.

Author contribution statement Phenotyping of Scots pine trees was done by MM and AK; RNA extraction, cDNA analysis, and qPCR analysis were performed by MM and AK; terpenoid analysis was carried out by RJ, MK, and JH; MM and AK analyzed the data; MM and AK prepared the manuscript draft; MM, AK, and FA conceived the study, contributed to the experimental design and interpretation of the data. All authors read and approved the final version of the manuscript.

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Compliance with ethical standards

Conflict of interest Authors declare no conflicts of interest.

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